Catalytic Metallodrug Targeting of MMP-2 and MMP-9

A Senior Honors Thesis

Presented for the fulfillment of the requirements for graduation with research distinction in Chemistry in the undergraduate college of Natural and Mathematical Sciences at The Ohio State University

By

Tyler Church

The Ohio State University
June 2012

Project Advisor: Professor James A. Cowan, Department of Chemistry

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ABSTRACT

Metastasis is the fatal weapon for most cancer. Through access to the vascular and lymphatic systems, cancer is able to spread to vital organs throughout the body. This is made possible by the degradation of basement membranes and cleavage of the extracellular matrix. Matrix metalloproteinases are the enzymes that are responsible for both of the aforementioned tasks. Two matrix metalloproteinases, MMP-2 and MMP-9, are zinc dependent enzymes that have roles in tumor progression through their involvement with the degradation of the ECM. Specific enzymes such as these are viable anti-cancer targets. This thesis evaluates the use of a metallodrug, which consists of a metal binding motif, a linker, and a target recognition domain to target these enzymes. Carbonic anhydrase, a well-studied enzyme in the literature, was used as a model to design the metallodrugs. The results of this project show that the metallodrug shows promise as a therapeutic against carbonic anhydrase, and through modification could be used to inhibit and inactivate matrix metalloproteinases as well.

ACKNOWLEDGEMENTS

I would first and foremost like to thank my research advisor, Dr. James Cowan for giving me the opportunity to conduct research in his laboratory. I have learned a great deal from his mentorship and have developed critical thinking skills that will further my professional career within the sciences and medicine.

I thank Dr. Iraj Ghazi, Jeff Joyner, James Ross, Lalintip Hocharoen, Seth Bradford, Wenbin Qi, Jingwei Li, Jessica Marstellar, Insiya Fidai, John Buzzo, Alex Hardin, Zhen Yu, and Meika Travis for their mentorship, advice, and friendship while being a member of the Cowan research group.

I would like to thank the Pelotonia Undergraduate Fellowship Program and the Arts and Sciences Honors College for funding that made this project possible.

I thank Dr. Joshua Goldberger and Dr. Mark Seeger for being a part of my thesis review committee.

Finally, I would like to thank my family for their continual and unconditional love and support. They have provided me with far more emotional and financial support than I could ever have asked of them. Without them, my undergraduate education at The Ohio State University would not have been a success.

INTRODUCTION

Tumor progression and metastasis is the fatal step for most cancer. Metastasis is the cause of 90% of solid tumor fatalities[1]. Cancer originates as a single tumor and is spread throughout the body utilizing the vascular and lymphatic systems created through angiogenesis. Angiogenesis is the creation of new blood vessels from old or pre-existing blood vessels. This physiological process is essential in wound healing as well as in the metastasis of cancer. Many enzymes play an active role in tumor progression. One such family, matrix metalloproteinases have shown proteolytic activity linking them to this progression.

The matrix metalloproteinase family currently has 24 structurally related members. The most explored link to cancer is their function of breaking down and cleaving components off of the extracellular matrix (ECM) as well as other basement membranes. Through this function, cancer cells have access to entering the stromal matrix. The activity of these enzymes are not just limited to the degradation of the ECM, but also reduction of apoptosis in cancer cells, destruction of chemokine gradients, and activation of various growth factors[2].

Much work was originally put towards development of MMP inhibitors (MMPIs). Many of these passed early evaluation and immediately went into clinical trials where poor results proved discouraging. However, most of these early tests were conducted using broad-spectrum inhibitors, inhibiting multiple MMP's. Certain MMP's demonstrate a more positive correlation between proteolytic activity and

cancer progression. The initial early trials were focused on late and advanced stage cancer patients, whereas early studies showed that MMPI's would be most effective with early stage patients[3].

Not all early trials produced discouraging results. One such trial involved patients with gastric carcinoma who were treated with marimastat resulting in an increase in patients that survived two or more years[3]. New approaches that are more selective compared to old methods should have increased success. Targeting specific MMP's and their substrates can accomplish this.

This project looks at specifically targeting MMP-2 and MMP-9. These two matrix metalloproteinases are both correlated with cancer progression. Tumor angiogenesis requires the degradation of the basement membrane and extracellular matrix surrounding blood vessels, followed by chemotaxis of endothelial cells, the proliferation of endothelial cells, and finally reformation of the basement membrane along with new blood vessel growth[4].

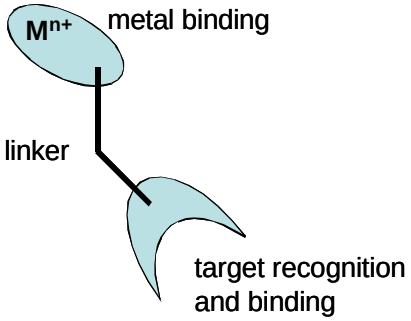
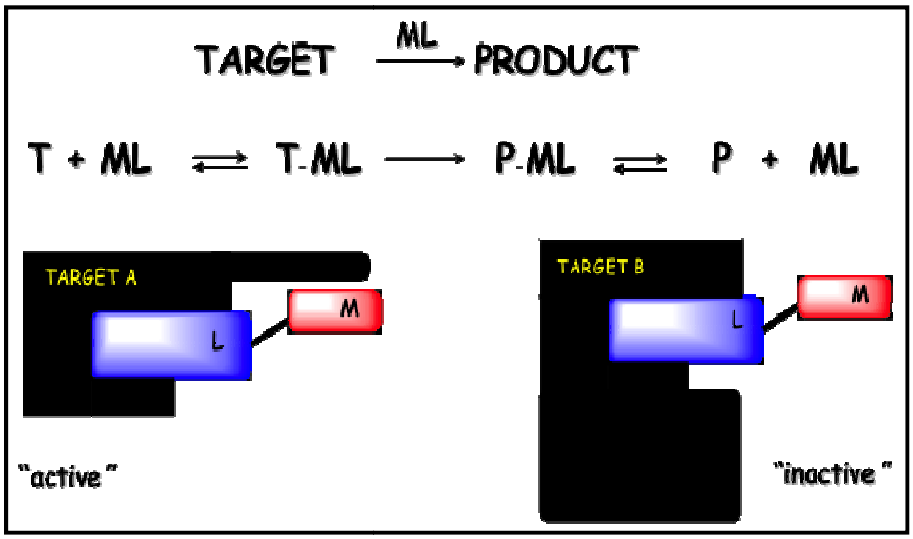


Fig. 1. Metallodrug Design. The metal binding and targeting domains may be directly linked or contain a linker.

Traditional drugs display high affinity, but reversible binding to a therapeutic target. The goal is to inhibit the function of the target (normally a protein). However, activity is

retained and can be manifested if the concentration of drug falls below a minimal level. By contrast, catalytic metallodrugs promote the irreversible destruction of target RNAs or proteins[5,6,7,8,9]. The metal binding domain catalyzes redox chemistry and/or peptide modification, while the target recognition domain promotes the expected specificity (Fig. 1). While such molecules retain traditional inhibitory (binding) properties, they also have the capability for catalytic inactivation of target molecules (Fig. 2).

Figure 2. Metallodrug Reaction Scheme



Irreversible destruction of a target affords the potential for:

- sub-stoichiometric administration of drug,
- significant reduction of dosage with a commensurate decrease or elimination of side effects or toxicity, and
- Also affords less risk for the development of drug resistance.

These key points differentiate the activity of catalytic metallodrugs, from high affinity binding that is essential for the classical inhibitory mechanism of drugs currently on the market. High affinity binding of the targeting domain may not be desirable from the viewpoint of facile release of the metallodrug following inactivation of the target. Optimization of the binding affinity of the targeting domain is an issue that will need to be considered on a case-by-case basis. High affinity binding to the target clearly has desirable traits, but may be unnecessary with the catalytic metallodrug concept described here.

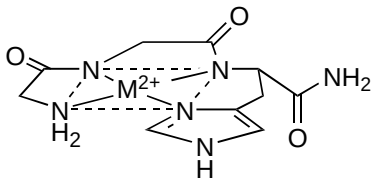


Fig. 3. Structure of amidated M•Gly-Gly-His.

Low molecular weight compounds could be cleared quickly from the serum. A peptide design for ligands that could, if necessary, be extended to the formation of fusion proteins (joining the ATCUN motif (Fig. 3) to a larger stabilizing protein partner) with the active peptide located at the N-terminus through a peptide linker, or in the case of non-peptide molecules by conjugation to other large stabilizing polymers. The clearance of such high molecular weight molecules would be considerably slower. To deal with potential instability toward protease digestion, the introduction of D-amino acid forms could be instituted.

The enzyme carbonic anhydrase is well characterized throughout the literature. Carbonic anhydrase helps to maintain the pH of the blood as well as to rid tissue of

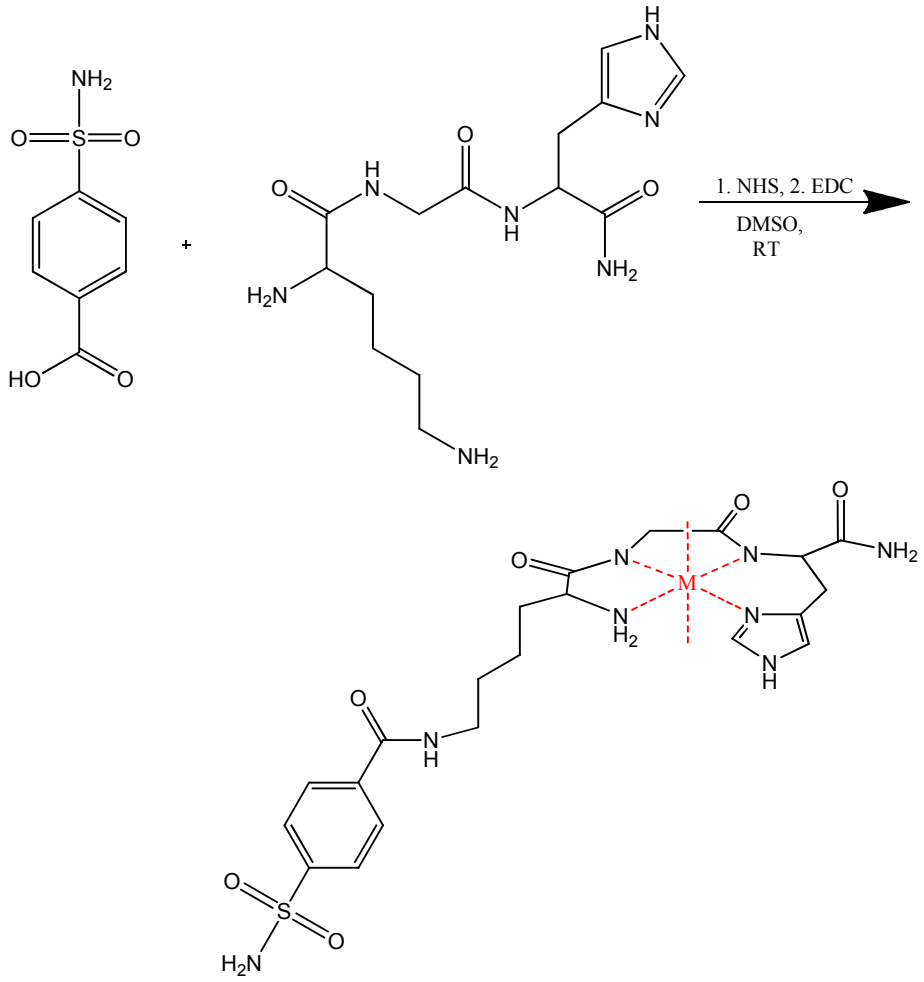
carbon dioxide. It accomplishes this task by converting carbon dioxide and water to bicarbonate and protons. It also is useful in the reverse of this task in converting protons and bicarbonate into carbon dioxide and water. Carbonic anhydrase is a zinc dependent enzyme, just like MMP-2. Literature has found similarities between the active sites and thus many common inhibitors[10]. This allows carbonic anhydrase to serve as a cost effective model system for studying inhibitors as was conducted in this study.

EXPERIMENTAL AND DATA ANALYSIS

Synthesis of Therapeutic Agents

The targeting domain, 4-carboxybezene sulfonamide was obtained from Sigma Aldrich. The amidated peptide, KGH was obtained from NEO Biosciences. The peptide was quantified through the use of a metal titration to ensure stock quantity. DMSO was first dried prior to use through the use of molecular sieves. The targeting domain, 4-carboxybenzene sulfonamide was dissolved in 60-µL DMSO at a concentration of 100 mM. Next, KGH was then added to this solution for a concentration of 100 mM as well. Next, N-hydroxysuccinimide (NHS) was added to the solution for a concentration of 200 mM NHS. The final reagent, the carbodiimide EDC was added at a concentration of 200 mM to the solution. The reaction was allowed to react at room temperature for 24 hours.

Figure 4. Coupling Reaction Scheme (KGH-amide + 4-carboxybenzene sulfonamide)



Following the 24 hour reaction period, the reaction was diluted to 600 µL with nano-pure water to ensure that the DMSO concentration was 10%. The completed reaction was then separated by High Performance Liquid Chromatography (HPLC). A reverse phase C18 monomeric column was used. The A-eluent was water with 0.1% TFA. The B-eluent was acetonitrile with 0.1% TFA. The eluent gradient can be seen below in figure 5.

Fig. 5. HPLC Gradient

Time (min)	0	2	3	10	50	60	70
%B	0	0	10	10	40	100	100
%A	100	100	90	90	60	0	0

The wavelength 280 nm was monitored as this is where the aromatic ring of the sulfonamide can be observed. The collected aliquots were lyophilized and then analyzed by ESI mass spectrometry.

Buffer Preparation

Two different Tris buffers were used throughout the compound synthesis and enzyme assays. The first contained 12.5 mM Tris-Hydrochloride and 50 mM NaCl that was adjusted to a pH of 7.4. The second buffer contained 50 mM Tris Hydrochloride and 150 mM NaCl that was adjusted to a pH of 7.4.

Nickel Titration

The compound was then suspended in 500 µL of Tris buffer (12.5 mM, 75 mM NaCl) and analyzed for concentration through a nickel titration. The estimated concentration of the solution was around 1 mM. The compound was diluted ten-fold using Tris buffer (12.5 mM, 75 mM NaCl). Nickel chloride (1 mM) was titrated into the solution in 2 µL increments. The increment was mixed and allowed to incubate for five minutes. An absorbance scan was then run from 200 to 600 nm. Points from

absorbance at 245 nm were fit to quantify the concentration. The inflection point was regarded as the concentration in which nickel to ligand was in a one to one ratio.

CA-II Binding Assay

The K_m of hydrolysis of 4-nitrophenyl acetate by carbonic anhydrase is 3.88 mM[8]. The IC-50 value was first determined for the targeting compound, 4-carboxybenzene sulfonamide. The activity of the enzyme at a concentration of 2 μM per individual run was monitored for two minutes at 400 nm in the presence of 4-carboxybenzene sulfonamide between the concentrations of 0.04-80 μM for two minutes. The slope of the initial velocity for each concentration was found and fit to a logistic equation to find the IC-50 values. This same procedure was done for the coupled product 4-carboxybenzene sulfonamide KGH, except that the concentration of inhibitor used ranged from 0.4-13.5 μM. The coupled product was also incubated with equal amounts of copper for 30 minutes and run with the same concentrations. Controls of free copper and nickel were also run to find IC-50 values. The concentration of copper (II) chloride used ranged from 10-1000 μM. The range of nickel (II) chloride ranged from 100-1500 μM.

Enzyme Inactivation

The inactivation reaction of the enzyme was conducted in 100 μL Tris buffer (50 mM, 150 mM NaCl) with CA-II (7.5 μM), Cu-KGH 4-carboxybenzene sulfonamide product (0.605 μM), hydrogen peroxide (1 mM), and ascorbate (1 mM). The copper was allowed to incubate with KGH 4-carboxybenzene sulfonamide for 30 minutes

prior to addition. From this reaction, 10 μL was taken and combined in a cuvette with 90 μL of 4-nitrophenyl acetate (1.1 mM) in Tris buffer (50 mM, 150 mM NaCl). The reaction was monitored at 400 nm at 1, 10, 20, 40, 60, 90, and 120 minute increments. The following controls were run simultaneously:

1. Enzyme and Substrate
2. Enzyme, Substrate, and Catalyst
3. Ascorbate, Hydrogen Peroxide, and Substrate
4. Enzyme, Substrate and Catalyst.

The slope of each initial velocity from each two minute run was found and fit to a graph.

MMP-2 Buffer and K_m Determination

The buffer used with MMP-2 contained 50 mM Tris, 10 mM $CaCl_2$, 150 mM NaCl, and 0.05% (w/v) Brij 35. The pH was adjusted to 7.5. The K_m was measured by incubating a fixed concentration of enzyme (1.41 nM) with varying concentrations of substrate (1-50 μM). The fluorogenic peptide substrate, MCA-Pro-Leu-DPA-Ala-Arg-NH_2 was acquired from R&D Systems. The K_m was measured using a fluorescence plate reader and 96 well plates. The solution was excited at 300 nm for an emission at 405 nm. The reaction was allowed to run for 60 min, but the slope of the initial velocity was found from the first eight minutes of the reaction.

MMP-2 Binding Assay

An IC-50 assay was run for 4-carboxybenzene sulfonamide with MMP-2. Similar concentrations of inhibitor that were used for CA-II were used in this assay (0.01-100 μM) with a fixed concentration of MMP-2 (1.41 nM). The reaction was excited at 300 nm and the emission at 405 nm was measured. The slope of the velocity after eight minutes was plotted on a graph.

RESULTS

The coupling reaction of 4-carboxybenzene sulfonamide resulted in a yield of 7.8%. This produced a sufficient amount of inhibitor to run the chemical analysis. The separation by HPLC can be seen in figure 6. The product came out of the HPLC between 24.6 and 25.6 minutes. The lyophilized product was then further analyzed by ESI. The peak at 523.3 and 262.1 correspond to the product. The ESI spectrum can be seen in figure 7. Following re-suspension the product concentration was 0.93 mM. The titration was conducted using a ten-fold dilution and can be seen in figure 8.

Figure 6. HPLC Separation

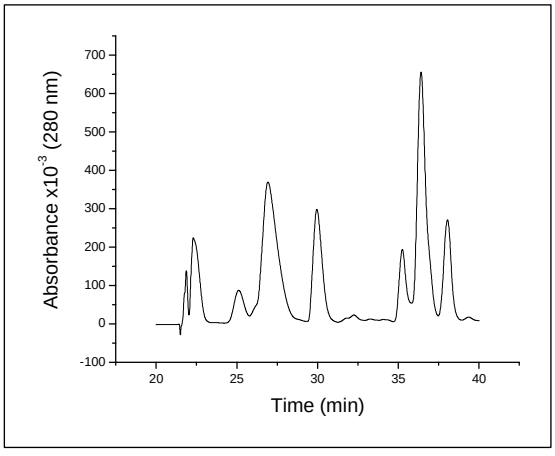


Figure 7. ESI Analysis

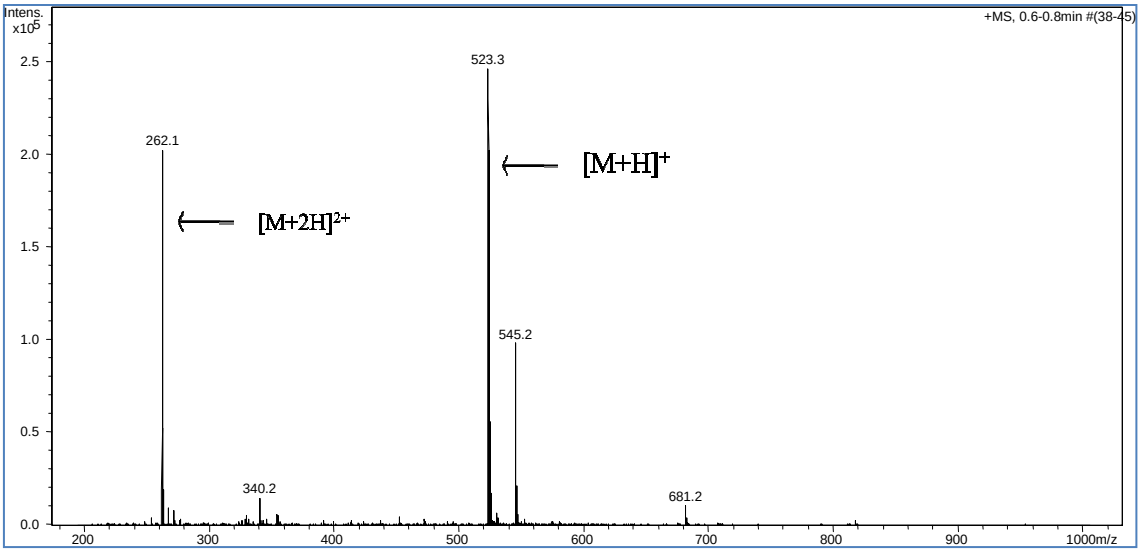
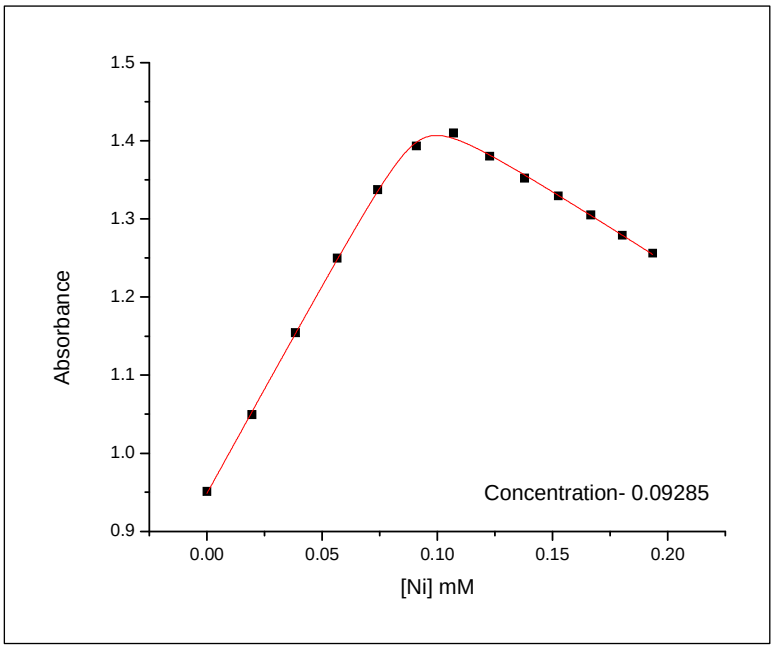


Figure 8. Determination of Product Concentration by Nickel Titration



The IC-50 value for 4-carboxybenzene sulfonamide was found to be 0.31±0.02 μM. The IC-50 value for the metal free KGH 4-carboxybenzene sulfonamide was found to be 1.07±0.09 μM. The IC-50 value for Cu-KGH 4-carboxybenzene sulfonamide was

found to be 1.8±0.3 μM. Graphs displaying the IC-50 values can be found in figures 9, 10, and 11. The IC-50 value for copper was found to be 87±7 μM. The IC-50 value for nickel was 2400±300 μM. The IC-50 data for the free metal controls can be found in figure 13.

Figure 9. IC-50 4-carboxybenzene sulfonamide

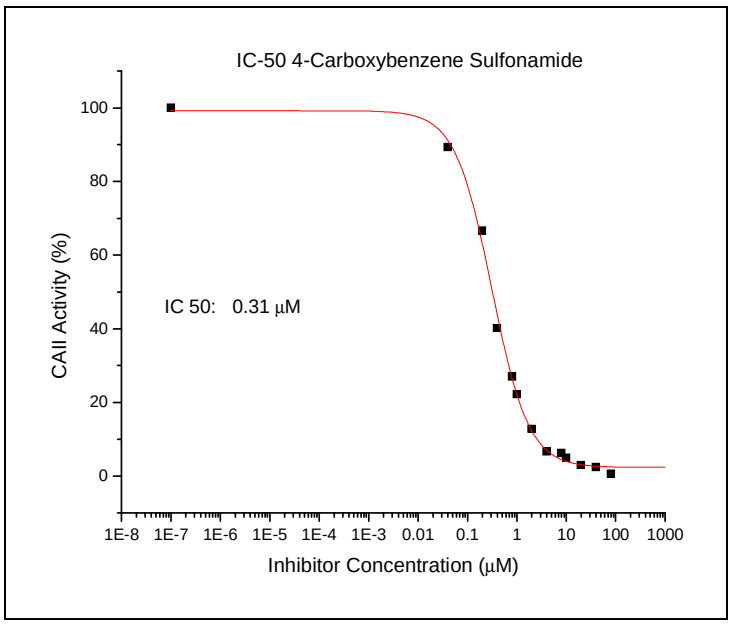


Figure 10. IC-50 Metal Free KGH 4-carboxybenzene sulfonamide

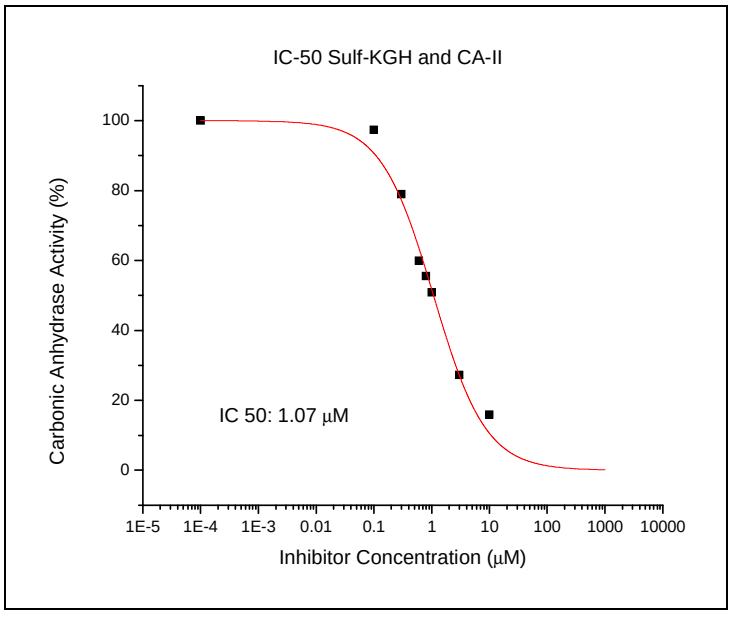


Figure 11. IC-50 Cu-KGH 4-carboxybenzene sulfonamide

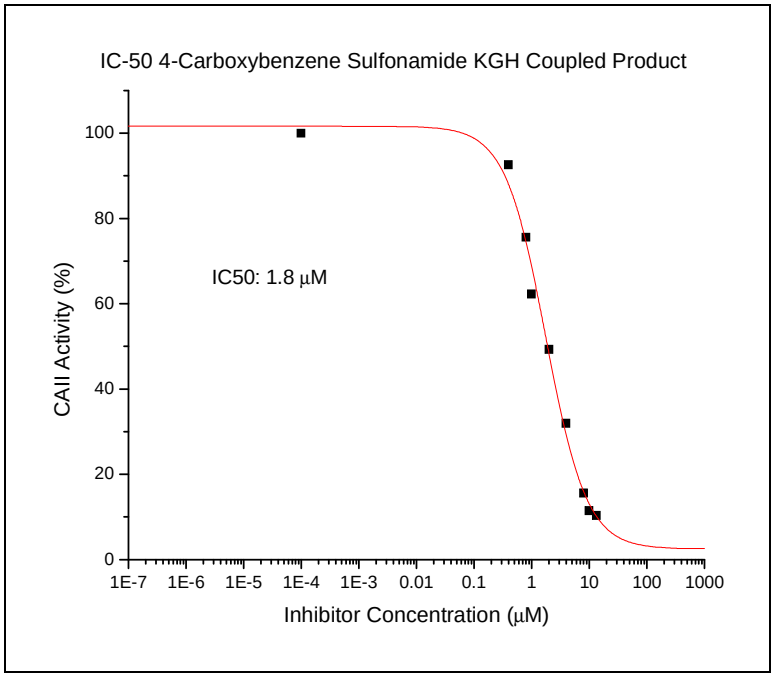
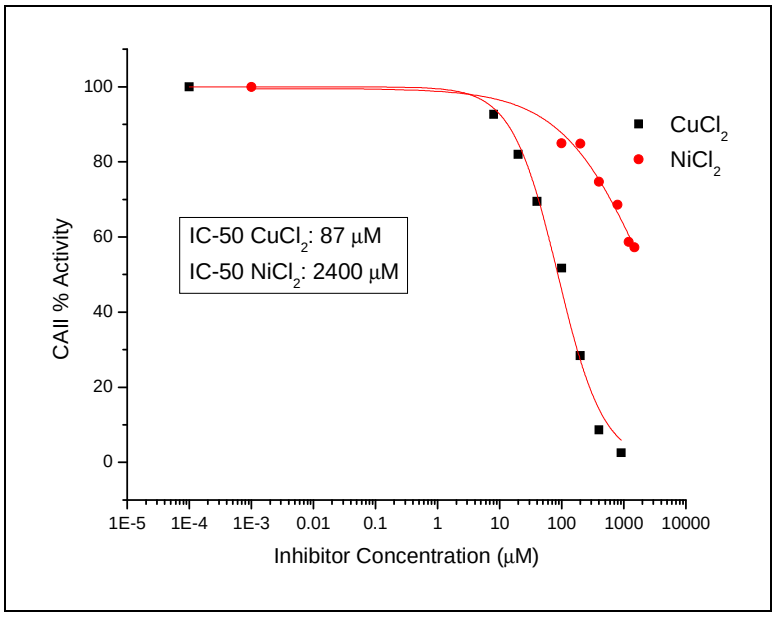


Figure 12. IC-50 Free Metal Controls



The time dependent inactivation with the metal bound catalyst resulted in a two-fold increase in inactivation when compared to the co-reagents. The initial velocities

for the assay containing the enzyme, substrate, hydrogen peroxide, ascorbate, and Cu-KGH 4-carboxybenzene sulfonamide can be seen in figure 14. The slopes of the time dependent inactivation of the enzyme along with the controls is plotted in figure 14.

Figure 13. Initial Velocities of the Time Dependent Inactivation of CA-II

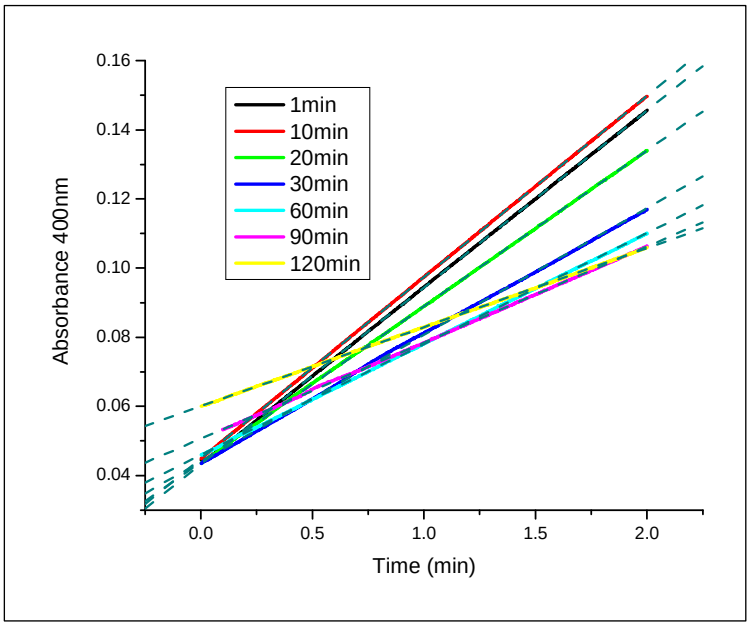
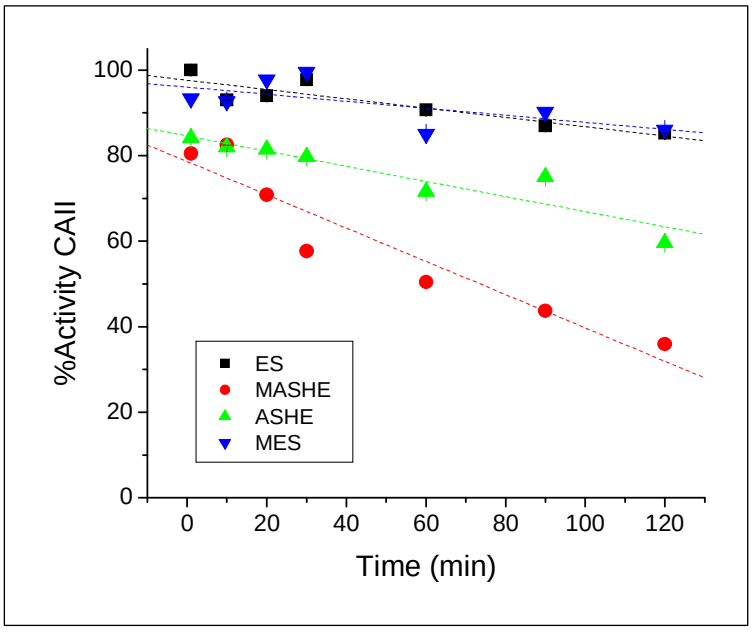


Figure 14. Time Dependent Inactivation of CA-II



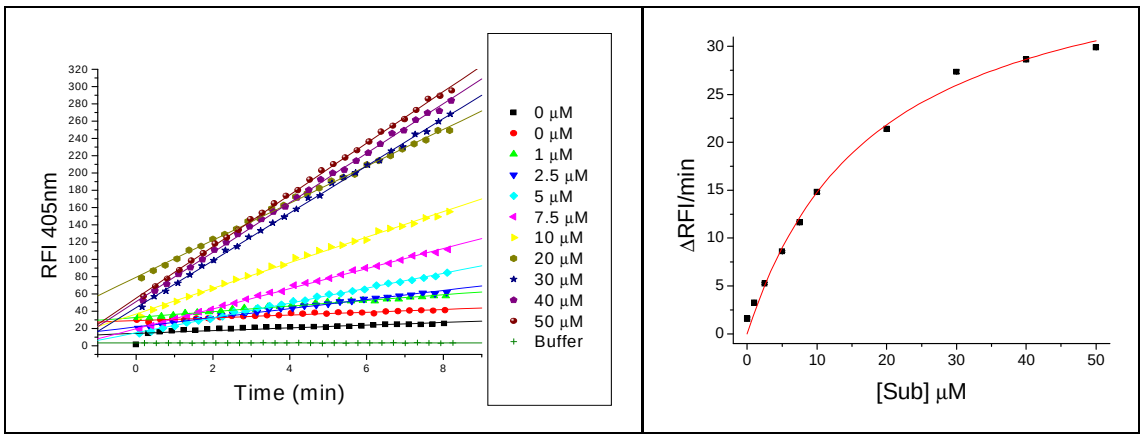
Key

- E- Enzyme
- S- Substrate
- M- Catalyst
- H- Hydrogen Peroxide
- A- Ascorbate

	Initial Slope (-%Activity/min)
E+S	0.11±0.02
M+A+H+E+S	0.39±0.06
A+H+E+S	0.18±0.03
M+E+S	0.08±0.04

The K_M of MMP-2 was found to be 18±2 µM. The initial velocities as well as the change in relative fluorescence over time can be seen in figure 15.

Figure 15. K_M Determination

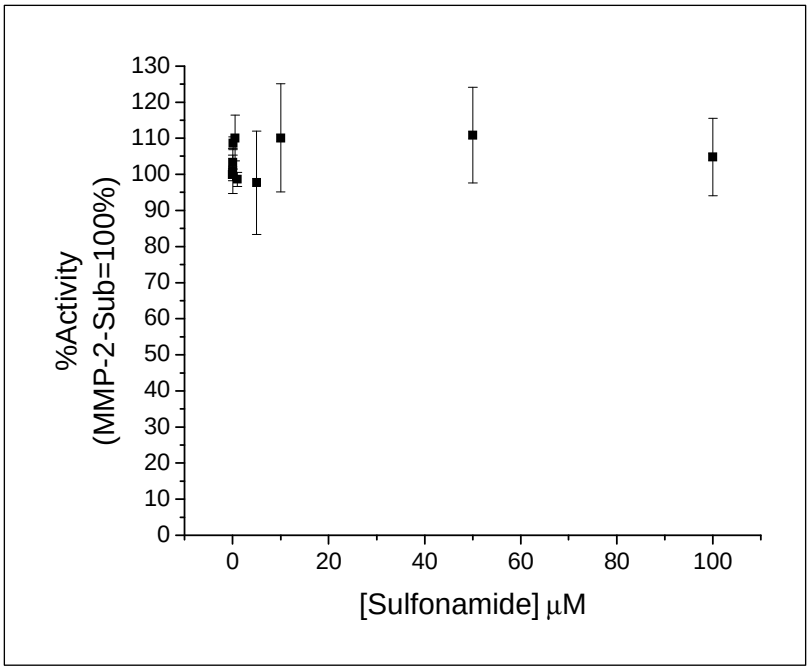


V_{max} 41± 2 ΔRFI/min

K_m 18± 2 µM

Binding assays were run with 4-carboxybenzene sulfonamide and MMP-2. IC-50 values could not be elucidated in the µM range. This data can be seen in figure 16.

Figure 16. IC-50 MMP-2 and 4-carboxybenzene sulfonamide



DISCUSSION

The preliminary tests of compounds with carbonic anhydrase produced promising data of an effective, competitively binding compound. The binding did not change extensively from the targeting domain to the coupled compound indicating that the metal domain did not produce negative steric interactions within the active site. The IC-50 of the coupled Cu-KGH 4-carboxybenzene sulfonamide product was 1.8 μM. In the presence of peroxide and ascorbate, a decrease in enzyme activity is seen. This is further enhanced in the presence of the metal complex, a two-fold enhancement. It is interesting that the metal complex needs the co-reagents present. The initial inactivation of just the metal complex returns to baseline without the presence of co-reagents. A potential mechanism can be hypothesized from these results. In the presence of just peroxide and ascorbate, it can be hypothesized that hydroxyl radicals or other reactive oxygen species are produced, but with the addition of the metal complex, it either further facilitates this production or the chemistry is more mediated from the metal to the enzyme. Future studies by mass spectrometry could help to elucidate a mechanism for inactivation of this process.

The IC-50 for 4-carboxybenzene sulfonamide for carbonic anhydrase did not carry over to MMP-2. As evidenced by figure 12, competitive binding was not observed. This could be due to a different method or location of binding within the MMP-2 enzyme. This suggests that the binding of 4-carboxybenzene sulfonamide to carbonic anhydrase may utilize a different structure that is not present within the

active site of MMP-2 or is sterically blocked. Although this project did not determine any effective inhibitors for MMP-2, it paved the way in answering many initial questions about the design of a possible metallodrug against MMP-2.

Future work could utilize this same design with different comparative inhibitors. Literature indicates that hydroxamates have shown effective inhibition of MMP-2. This may be a future avenue to pursue as a possible targeting domain[10].

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